Docket No.: 0230-0233PUS1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Patent Application of:
Yuji ISHIDA

Application No.:	10/567,866	Confirmation No.:	2073
Filed:	September 21, 2006	Art Unit:	1638
For:	METHODS OF TRANSDUCING GENE INTO PLANT MATERIAL	Examiner:	David T. Fox

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Yuji Ishida, Ph.D. declare the following:

1. I am an inventor of the present application

2. I am a Japanese citizen employed as a Research Scientist with the Plant Innovation Center of Japan Tobacco, Inc., which is located at 700 Higashibara, Iwata, Shizuoka, Japan.

3. I graduated from the Shizuoka University in 1984. I received my Ph.D. from Gifu University. My profession experience is in the field of plant tissue culture and transformation.

4. I have reviewed and considered the Office Action, which issued on December 4, 2009, and the references cited therein including European Patent Application No. 1,306,440 to Hiei *et al.*, ("Hiei I"), European Patent Application No. 1,306,441 to Hiei II *et al.*, and Cheng *et*

al., *Plant Cell Reports*, 1996, 16:127-132, ("Cheng").

5. Described herein below are data, which demonstrate that pressurizing plant material using the methods described in the instant claims, including pressurizing plant material with syringes held by a tightened clamp, unexpectedly results in an increase in gene transfer efficiency without wounding the plant material. These results could not have been expected by an ordinary artisan from the cited references.

6. Experimental Evidence I. Study of Pressure-Induced Damage to Test Materials

The Claimed Methods Do Not Result In Wounding of Plant Material

I performed or had performed the following experiments under my supervision. The journal articles referenced below are cited in the enclosed Appendix. These experiments demonstrate that pressurizing plant material using the methods described in the present claims, such as with syringes and tightened clamps do not result in plant wounding.

6a. Materials and Methods

Test material

Suspension-cultured tobacco BY-2 cells (Nagata *et al.*, 1992) were sub-cultured and maintained in LSD liquid medium (LS inorganic salts modified to contain 1.65 g/l NH_4NO_3, 1.90 g/l KNO_3, 0.34 g/l KH_2PO_4, 0.44 g/l $CaCl_2 \cdot 2H_2O$ and 0.37 g/l $MgSO_4 \cdot 7H_2O$ as major salts, 1 mg/l Thiamine HCl, 100 mg/l myo-Inositol, 0.2 mg/l 2,4-D, 30 g/l Sucrose, pH 6.0) and provided for experiments on day 3 of subculture.

Pre-treatment

A micropipet chip with a tip that was narrowed by heating was inserted into the tip of each disposable syringe (10 ml volume). The chip was cut off at an appropriate length, and the tip was covered with parafilm. The syringes were filled with 4.8 milliliters (ml) suspension-cultured cells. The syringes were assembled and held with a clamp, followed by closing the clamp to increase the internal pressure of the syringes. The syringes were allowed to stand at room temperature for 15 minutes while being maintained at 10 atmospheres (or "atm") pressure (normal pressure + 9 atmospheres). The intensity of pressure was calculated from a percent (%) decrease in air volume within the syringes (pressurized group).

Eppendorf tubes (2 ml volume) were filled with about 1.5 ml cell suspension. The tubes were immersed in a washing vessel containing water and treated with an Ultrasonic Generator (U0150FS, Shinmeidai kogyo, Japan) for 2 seconds (ultrasonicated group).

Tubes (5 ml volume) were filled with about 5 ml cell suspension and allowed to stand at room temperature for 15 minutes. The cells thus prepared were used as control cells (untreated group).

Cell staining

The cell suspension after each treatment and the control cell suspension were sampled in 10 μl volumes. Each sample was mixed with an equal volume of 0.2% phenosafranin and observed under a microscope to count red-stained dead cells and non-stained living cells (400 or more cells in total).

6b. Results

The cells maintained at 10 atmospheres pressure for 15 minutes showed a survival rate of 93%, which was comparable to the survival rate of the control cells receiving no pre-treatment. In contrast, the cells ultrasonicated for 2 seconds showed a survival rate of 28%, and 70% or more of the cells were dead (Table-1).

It has been reported that ultrasonicated plant tissues are injured and, when inoculated with *Agrobacterium*, achieve a higher efficiency of gene transfer than achieved in untreated tissues (Trick and Finer 1997, 1998, Santarén *et al.* 1998). In the case of the cultured tobacco cells ultrasonicated in this experiment, not only the cells were injured, but also ultrasonication caused death of 70% or more of the cells. In contrast, the cells maintained at 10 atmospheres pressure for 15 minutes showed a survival rate comparable to that of the untreated cells, and there was no cell death caused by treatment under pressure. These results suggest that treatment under pressure causes very little damage to plant cells and has little effect on cell injury when compared to ultrasonication, which is generally considered to increase the gene transfer efficiency.

Table-1 Survival rate of cultured tobacco cells after each treatment

	Cell counts			
Treatment	Total	Living cells	Dead cells	Survival (%)
Control (untreated)	401	376	25	94
Pressurized	410	383	27	93
Ultrasonicated	407	112	295	28

7. Based upon the foregoing, injury (damage) to plant cells resulting from pressure treatment using syringes by a clamp and tightening the claims is not significant in comparison to cells, which are wounded by sonication. Accordingly, the claimed method does not result in plant wounding.

8. Further, an ordinary artisan would have recognized that pressurizing plant material by supplying a gas into a vessel containing a plant tissue or submerging a plant tissue bag sealed against the outside air in a liquid also would not cause wounding in plant material.

9. Experimental Evidence II Study of gene transfer under pressurized conditions

Pressurization results in an Increase in Gene Transfer Efficiency

The following experimental evidence demonstrates that pressurizing plant material according to the methods described in the present claims results in an increase in gene transfer efficiency in comparison to gene transfer efficiency when pressurization is not used.

The following experimental evidence further provides supplemental data that demonstrate that the increased gene transfer efficiency is observed across the pressure ranges and time periods of pressurization specified in the instant claims. That is, the working examples in the originally filed application provide data that show an increase in gene transfer efficiency at 2.4 atmospheres (+ 1.4 atmospheres), 15minutes, 4.2 atmospheres (+ 3.2 atmospheres), 15 minutes, 7.6 atmospheres (+ 6.6 atmospheres), 15 minutes, *see* Example 1(6). In addition, the increase in gene transfer efficiency is observed at 7.6 atmospheres (+ 6.6 atmospheres), 1 seconds, 7.6 atmospheres (+ 6.6 atmospheres), 3 seconds, 7.6 atmospheres (+ 6.6 atmospheres), 5 seconds, and 7.6 atmospheres (+ 6.6 atmospheres), 60 seconds, *see* Example 1(7). The data below demonstrate that pressurization at 6 atmospheres, 1 second and 8 atmospheres, 30 minutes, 2 atmospheres, 15 minutes and 10 atmospheres, 15 minutes also results in an increased gene transfer efficiency in comparison to the gene transfer efficiency when no pressure is applied to plant material.

9a. Material and Methods

Treatment under pressure

A micropipet chip whose tip was narrowed by heating was inserted into the tip of each disposable syringe (10 ml volume). The chip was cut off at an appropriate length, and the tip was covered with parafilm. The syringes were filled with 4.8 ml sterile distilled water, and aseptically collected immature embryos (variety: Yukihikari) were introduced thereinto. The syringes were assembled and held with a clamp, followed by closing the clamp to increase the internal pressure of the syringes. The syringes were allowed to stand at room temperature

while being maintained under pressurized conditions. The intensity of pressure was calculated from a percent decrease in air volume within the syringes. For a control, an almost equal number of immature embryos were introduced into Eppendorf tubes (2 ml volume) containing sterile distilled water and allowed to stand at room temperature.

Inoculation

The syringes were released from the clamp, and the immature embryos in the syringes were transferred to Eppendorf tubes (2 ml volume) containing liquid medium. *Agrobacterium tumefaciens* super-binary vector LBA4404 (pSB134) (carrying the HPT gene fused to a ubiquitin intron driven by a maize ubiquitin promoter and the GUS gene fused to a castor bean catalase intron driven by the cauliflower mosaic virus 35S promoter in the T-DNA region), which had been cultured for 3 days on AB medium containing 50 mg/l hygromycin and 50 mg/l spectinomycin, was scraped with a platinum loop and suspended at a concentration of about 10^9 cfu/ml in 1 ml liquid medium containing 100 μM acetosyringone. The immature embryos in the liquid medium were plated on nN6-As medium (N6 inorganic salts, N6 vitamins, 0.5 g/l casamino acid, 0.5 g/l L-proline, 1 mg/l 2,4-D, 0.5 mg/l NAA, 0.1 mg/l 6BA, 20 g/l sucrose, 10 g/l glucose, 10 μM $AgNO_3$, 100 μM acetosyringone, 8 g/l agarose, pH 5.2) with the embryonic disc facing upward. The inoculum (5 μl) was added dropwise onto the immature embryos. After the inoculum thus added dropwise was dried, the immature embryos were each plated at another site on the co-culture medium with the embryonic disc facing upward. The embryos were cultured at 25°C in the dark for 6 to 7 days.

GUS Assay

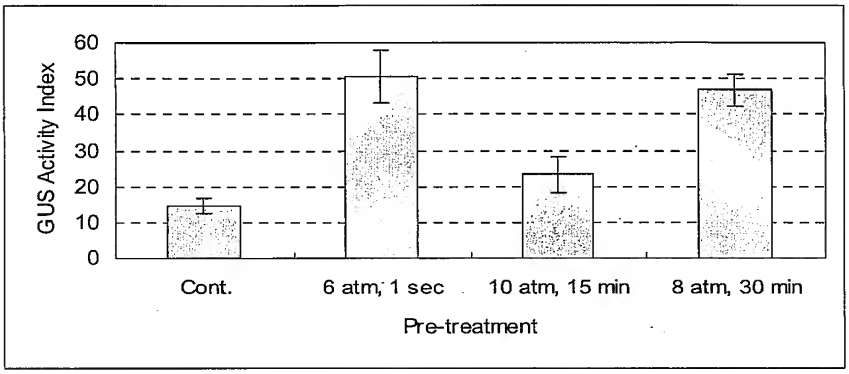
The co-cultured immature embryos were immersed in 0.1 M phosphate buffer (pH 6.8) containing 0.1% Triton X-100 and allowed to stand at 37°C for 1 hour. *Agrobacterium* was removed with the phosphate buffer, followed by addition of another phosphate buffer containing 1.0 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) and 20% methanol. After incubation at 37°C for 24 hours, blue-stained tissues were observed under a microscope.

The immature embryos were scored for GUS activity according to the ratio of GUS gene-expressing sites in the embryonic disc. Namely, a score of 0 was given for 0% expression, 2.5 for 1-5% expression, 7.5 for 5-10% expression, 17.5 for 10-25% expression, 37.5 for 25-50% expression, 62.5 for 50-75% expression, and 87.5 for 75-100% expression.

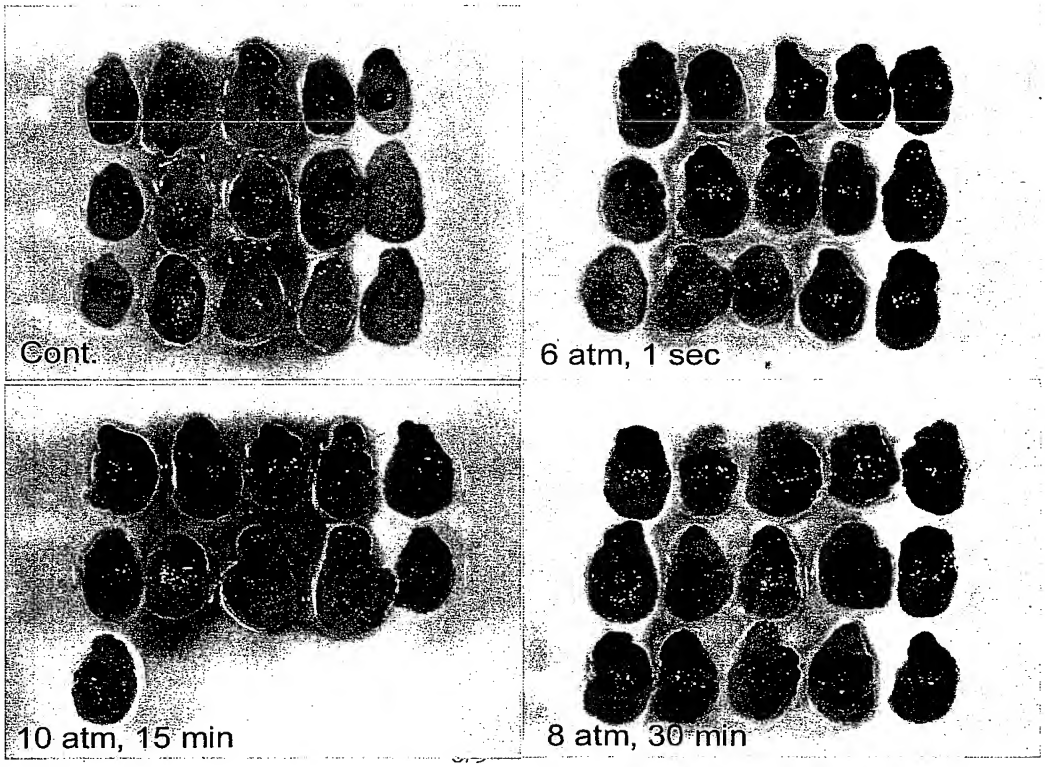
9b. Results

Before inoculation with *Agrobacterium*, immature embryos were treated at 6 atmospheres for 1 second, at 10 atmospheres for 15 minutes, or at 8 atmospheres for 15 minutes. Control embryos were allowed to stand under normal pressure (at 1 atmosphere) for 15 minutes in sterile distilled water. 11 to 16 immature embryos were tested for each group. After inoculation with *Agrobacterium*, the embryos were cultured at 25°C in the dark for 6 days and then analyzed by GUS assay. GUS gene expression was observed over a wider range of sites in all the treated groups than in the untreated control immature embryos, indicating that all the pressurized conditions tested in this experiment stimulate gene transfer (Figures 1 and 2).

Figure 1

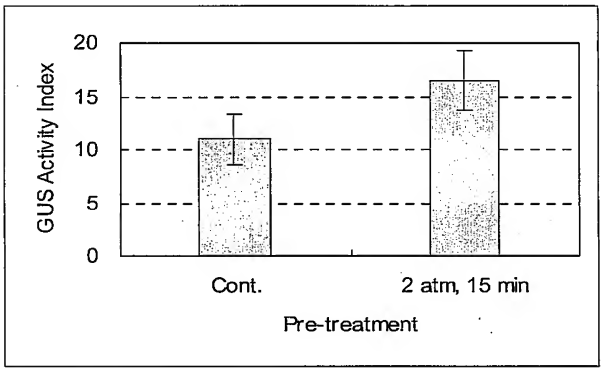


[Figure 2]

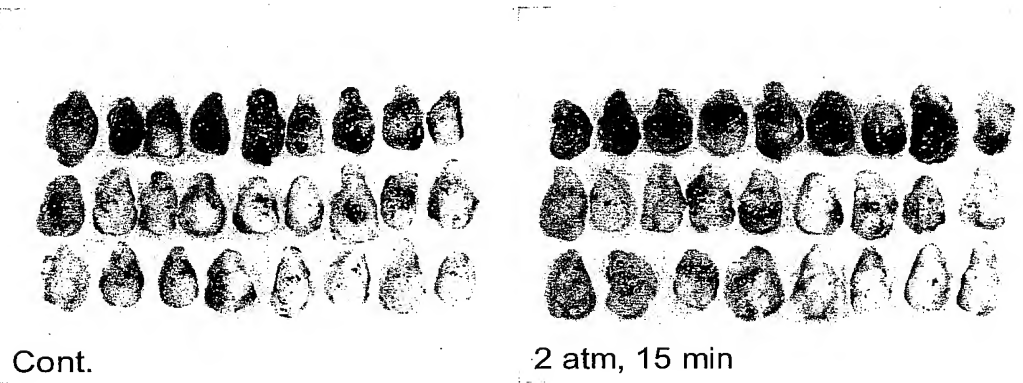


Before inoculation with *Agrobacterium*, the immature embryos were treated at 2 atmospheres for 15 minutes. Control embryos were allowed to stand under normal pressure (at 1 atmosphere) for 15 minutes in sterile distilled water. 26 immature embryos were tested for each group. After inoculation with *Agrobacterium*, the embryos were cultured at 25°C in the dark for 7 days and then analyzed by GUS assay. GUS gene expression was observed over a wider range of sites in the immature embryos treated at 2 atmospheres for 15 minutes than in the untreated control immature embryos, indicating that treatment at 2 atmospheres also produces the effect of stimulating gene transfer (Figures 3 and 4).

[Figure 3]



[Figure 4]



10. The experimental data submitted herein and in the working examples in the present application demonstrate that gene transfer efficiency is increased when the plant material is pressurized in comparison to no pressurization.

11. It is my opinion that an ordinary artisan at the time of the invention could not have reasonably predicted from the cited references that pressurization could have resulted in superior efficiency of gene transfer in plants in comparison to plants that are not pressurized. Further, this superior gene transfer efficiency is observed using methods that do not require wounding of the plant material. These benefits would not have been expected by an ordinary artisan at the time of the invention.

STATEMENT UNDER 18 U.S.C. § 1001

I declare that all statements made herein of my own knowledge are true and that all statements made herein on information are believed to be true. I further declare that the statements were made with knowledge that willful false statements and the like are punishable by fine or imprisonment or both under Section 1001, Title 18 of the United States Code.

Dated: March. 15. 2010

Respectfully submitted,

By Yuji Ishida

Yuji Ishida, Ph.D.

Enclosure: Appendix

APPENDIX

References

Nagata T, Nemoto Y, Hasezawa S (1992) Tobacco BY-2 cell line as the <HeLa> cell in the cell biology of higher plants. *Int Rev Cytol* 132: 1-30

Santarén ER, Trick HN, Essig JS, Finer JJ (1998) Sonication-assisted *Agrobacterium*-mediated transformation of soybean immature cotyledons: optimization of transient expression. *Plant Cell Rep* 17: 752-759

Trick HN, Finer JJ (1997) SAAT: Sonication-assisted *Agrobacterium*-mediated transformation. *Transgenic Res* 6: 329-337

Trick HN, Finer JJ (1998) Sonication-assisted *Agrobacterium*-mediated transformation of soybean [*Glycine max* (L.) Merrill] embryogenic suspension culture tissue. *Plant Cell Rep* 17: 482-488